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(54) Title: OLIGOPEPTIDES/EXPRESSION PRODUCTS OF THE AUJESZKY'S DISEASE VIRUS, AND RECOMBINANT POLYNUCLEOTIDES CODING THEREFOR

(57) Abstract

The invention relates to novel oligopeptides/expression products derived from glycoprotein I of the Aujeszky's disease virus, to their use for diagnostic purposes or for producing polyclonal or monoclonal antibodies, and to recombinant genetic information coding for the novel oligopeptides/expression products.

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Title: Oligopeptides/expression products of the Aujeszky's disease virus, and recombinant polynucleotides coding therefor.

This invention relates to novel oligopeptides/expression products which can be used for diagnostic purposes or for the production of polyclonal or monoclonal antibodies.

More specifically, the present invention relates to novel oligopeptides/expression products derived from glycoprotein I (referred to hereinafter by the conventional abbreviation gI) of the Aujeszky's disease virus (ADV, sometimes referred to as pseudorabies virus or PRV), and suitable for use in serological diagnostics directed to the differentiation of animals (specifically pigs) infected by the wild-type virus from animals not infected by the wild-type virus expressing the gI, more specifically animals vaccinated against ADV with a vaccin not containing or expressing gI (a gI-negative vaccin).

The present invention relates to these oligopeptides/expression products themselves, to immunogenic compositions containing one or more of these novel oligopeptides/expression products in a form suitable for immunisation purposes, to the use of the novel oligopeptides/expression products for diagnostic purposes or for producing polyclonal or monoclonal antibodies, and to recombinant genetic information (recombinant polynucleotides, i.e. recombinant DNA or recombinant RNA) coding for the novel oligopeptides/expression products.

It is known, for example, from European patent application EP-A-0 223 382 that the disease of Aujeszky is caused by the herpes virus PRV (or ADV). The virus causes huge losses in pig farms, especially due to fertility problems in sows, high mortality among piglets, and growth retardation among fatteners. All infected pigs form antibodies against the gI of ADV. These antibodies continue to be demonstrable in the blood for many years. Pigs vaccinated with gI-negative vaccins, on the other hand, do not form antibodies against gI of ADV.

To reduce economic losses, pigs are being vaccinated againt the disease on a large scale in many countries. However, vaccination alone is not sufficient to eradicate the ADV; the virus can continue to circulate in vaccinated pig populations. Therefore, to eradicate ADV in countries where vaccination is practised, it is of crucial importance to have a test to differentiate between infected and vaccinated pigs (in other words, pigs with antibodies against gI and pigs without antibodies against gI).

Tests for making such a distinction have been developed. These tests are based on a detection of antibodies against gI (Van Oirschot et al., J. Gen. Virol. 67, 1986, 1179-1182; Anonymous, Interviews 12, 1987, 25-32; Van Oirschot et al., J. Virol. Meth. 22, 1988, 191-206; Eloit et al., Vet.Rec. 124, 1989, 91-94) or a detection of antibodies against glycoprotein X (McMillen and McDonald, Proc. 1st Eur. Congr. Vet. Virol. 1989, p. 38).

An article by Petrovskis et al., J. Virol. 60, 1986, 185-193, shows that the full amino acid sequence of the glycoprotein gI is known. In the research on which the present invention is based, the sequence shown in this article has been used.

The above-mentioned European patent application EP-A-0 223 382 discloses recombinant DNA containing the genetic information coding for gI of PRV, which consists of 577 amino acids. Although it also refers to fragments of gI exhibiting "pseudorabies virus activity", it does not make clear which fragments of the gI have such "pseudorabies virus activity", or which fragments or parts of the gI are immunologically functional. Both for diagnostic purposes and for the production of polyclonal and monoclonal antibodies with a high specific affinity to gI, it may be useful to use a suitable fragment instead of the complete protein.

Up until now, no oligopeptides/expression products of the gI have been described which are capable of generating antibodies in the pig, the natural host of ADV. Such

oligopeptides/expression products could mean an important advance in the field of diagnostics. Such oligopeptides/expression products could be used for a diagnostic test to differentiate infected pigs from pigs vaccinated with gI-negative vaccins. Such oligopeptides/expression products could also be used for the development of a so-called "pig-side" test in accordance with the principle described in an article by Kempt et al., Science 241, 1988, 1352-1354. Such a test could be based upon a binding of the antigenic sequence of the oligopeptide/expression product by antibodies against the gI, present in infected animals. For such a test, the oligopeptide/expression product would have to be coupled to a monoclonal antibody directed against pig erythrocytes.

Another possibility would be for the oligopeptides/expression products to be used for generating an immune response within the framework of a process for producing polyclonal or monoclonal antibodies specific for the antigenic region(s) concerned, and, in turn, suitable for use in a diagnostic test.

According to the present invention, by means of the so-called PEPSCAN method of Geysen et al. (Proc.Natl.Acad. Sci. USA 81, 1984, 3998-4002; Proc.Natl.Acad.Sci. USA 82, 1985, 178-182; " Synthetic peptides as antigens", 1986, 130-149, Wiley; WO 84/03506 and WO 84/03564) and of monoclonal antibodies directed against the glycoprotein I of ADV, reactive sequences located on the glycoprotein I have been found which can be used for the above purposes in the form of oligopeptides/expression products.

The present invention is in the first place embodied in an oligopeptide having the following amino acid sequence (according to the three-letter code)

Ala Gly Asp Asp Asp Leu Asp Gly Asp Leu Asn Gly Asp Asp Arg
Arg Ala Gly Phe Gly Ser Ala Leu Ala Ser Leu Arg Glu Ala Pro
Pro Ala His Leu Val Asn Val Ser Glu Gly Ala Asn Phe Thr Leu
Asp Ala Arg Gly Asp Gly Ala Val Val Ala Gly Ile Trp Thr Phe

Leu Pro Val Arg Gly Cys Asp Ala Val Ala Val Thr Met Val Cys
Phe Glu Thr Ala Cys His Pro Asp Leu Val Leu Gly Arg Ala Cys
Val Pro Glu Ala Pro Glu Arg Gly Ile Gly Asp Tyr Leu Pro Pro
Glu Val Pro Arg Leu Gln Arg Glu Pro Pro Ile Val Thr Pro Glu
Arg Trp Ser Pro His Leu Thr Val Arg Arg Ala Thr Pro Asn Asp
Thr Gly Leu Tyr Thr Leu His Asp Ala Ser Gly Pro Arg Ala Val
Phe Phe Val Ala Val Gly Asp Arg Pro Pro Ala Pro Leu Ala Pro
Val Gly Pro Ala Arg His Glu Pro Arg Phe His Ala Leu Gly Phe
His Ser Gln Leu Phe Ser Pro

which sequence corresponds to that of the amino acids 52-238 of the glycoprotein I (gI) of the Aujeszky's disease virus (ADV), or an immunologically funetional fragment or derivative thereof.

The term "oligopeptide" is used herein in a broad sense, and, specifically, should not be construed as being in any way limitative with regard to the way in which it has been obtained. The term includes both peptides prepared by chemical synthesis, and products obtained by the expression of relevant genetic information in suitable (micro)biological systems. Therefore, to leave no doubt, the phrase "oligopeptides/expression products" is used in many places in the present text.

More specifically, the invention is embodied in an oligopeptide having the following amino acid sequence (according to the three-letter code)

Ala Gly Asp Asp Asp Leu Asp Gly Asp Leu Asn Gly Asp Asp Arg
Arg Ala Gly Phe Gly Ser Ala Leu Ala Ser Leu Arg Glu Ala Pro
Pro Ala His Leu

which sequence corresponds to that of the amino acids 52-85 of gI of ADV, or an immunologically functional fragment or derivative thereof.

As the oligopeptide in question is a relatively small one, chemical synthesis (by techniques known per se) will be a suitable way of obtaining the oligopeptide. Production by expression of relevant genetic information in a (micro) biological host, however, is also possible.

One possible embodiment of the present invention relates to an oligopeptide having the following amino acid sequence (according to the three-letter code)
Ala Gly Asp Asp Asp Leu Asp Gly Asp Leu Asn Gly Asp Asp Arg Arg
which sequence corresponds to that of the amino acids 52-67 of gI of ADV, or an immunologically functional fragment or derivative thereof.

Another possible embodiment of the present invention concerns an oligopeptide having the following amino acid sequence (according to the three-letter code)
Asn Gly Asp Asp Arg Arg Ala Gly Phe Gly Ser Ala Leu Ala Ser Leu Arg Glu Ala Pro Pro Ala His Leu
which sequence corresponds to that of the amino acids 62-85 of gI of ADV, or an immunologically functional fragment or derivative thereof.

A possibility within this embodiment is an oligopeptide having the following amino acid sequence (according to the three-letter code)
Ala Gly Phe Gly Ser Ala Leu Ala Ser Leu Arg Glu Ala Pro Pro Ala His Leu
which sequence corresponds to that of the amino acids 68-85 of gI of ADV, or an immunologically functional fragment or derivative thereof.

An important preferred embodiment of the present invention concerns an oligopeptide having the following amino acid sequence (according to the three-letter code)
Arg Glu Ala Pro Pro Ala His Leu Val Asn Val Ser Glu Gly Ala Asn Phe Thr Leu Asp Ala Arg Gly Asp Gly Ala Val Val Ala Gly Ile Trp Thr Phe Leu Pro Val Arg Gly Cys Asp Ala Val Ala Val Thr Met Val Cys Phe Glu Thr Ala Cys His Pro Asp Leu Val Leu Gly Arg Ala Cys Val Pro Glu Ala Pro Glu Arg Gly Ile Gly Asp Tyr Leu Pro Pro Glu Val Pro Arg Leu Gln Arg Glu Pro Pro Ile Val Thr Pro Glu Arg Trp Ser Pro His Leu Thr Val Arg Arg Ala Thr Pro Asn Asp Thr Gly Leu Tyr Thr Leu His Asp Ala Ser Gly Pro Arg Ala Val Phe Phe Val Ala Val Gly Asp Arg Pro Pro Ala

Pro Leu Ala Pro Val Gly Pro Ala Arg His Glu Pro Arg Phe His Ala Leu Gly Phe His Ser Gln Leu Phe Ser Pro

which sequence corresponds to that of the amino acids 78-238 of gI of ADV, or an immunologically functional fragment oir derivative thereof.

Here again, the oligopeptide can be produced by both chemical synthesis and expression. In the case of this oligopeptide, however, production by expression of relevant genetic information is much preferred.

The phrase "immunologically functional fragment or derivative" is used herein to designate parts or modified forms of the oligopeptide/expression product which are also reactive with antibodies against gI. Fragments of the oligopeptide will be at least eight to nine amino acids long for them to have a specific reactivity with antibodies against gI. In the case of derivatives of the oligopeptide, such modifications of the oligopeptide are permitted that the reactivity is not reduced below one-fifth of the reactivity of the oligopeptide. Modifications include, for example, replacing a limited number of amino acids by other amino acids, deleting or adding (e.g. inserting) a limited number of amino acids, and chemical modifications, such as the introduction of substituents at a limited number of amino acids and blocking the terminal amino and carboxyl groups. Expression products which in addition to a sequence according to the present invention comprise a foreign protein fragment (i.e., an amino acid sequence not occurring in gI, such as an amino acid sequence of a protein different from gI), in particular fusion proteins, are also covered by this terminology.

The oligopeptide according to the present invention can be synthesized by known per se methods. They can also be obtained, however, by expression (e.g. in bacteria or in eukaryotic cells) of recombinant DNA comprising nucleotide sequences coding for one or more oligopeptides according to the present invention.

Accordingly, the present invention also provides a recombinant polynucleotide comprising a nucleotide sequence coding for an oligopeptide or immunologically functional fragment or derivative thereof according to the present invention, as defined hereinbefore.

More specifically, the invention provides recombinant DNA comprising a DNA sequence coding for an oligopeptide or immunologically functional fragment or derivative thereof according to the present invention.

A recombinant DNA consisting of a vector portion and an insertion portion containing a DNA sequence coding for an oligopeptide or immunologically functional fragment or derivative thereof in accordance with this invention is preferred.

The present invention is further embodied in a host cell genetically engineered with the help of such a recombinant DNA to be given the capacity of expressing an oligopeptide or immunologically functional fragment or derivative thereof in accordance with this invention. The nature of the host cell is relatively uncritical, and is mainly determined by considerations of production engineering and the available technical possibilities. Generally speaking, microorganisms, in particular bacteria (e.g. E.coli) will be selected, but eukaryotic cells, such as yeast cells, fungi, mammalian cells and plant cells can also be used.

As stated before, the present invention also relates to expression products that can be obtained by using such transformed host cells.

Naturally, an application of the oligopeptides/expression products in a process for producing polyclonal or monoclonal antibodies requires the oligopeptides/expression products in question to be used in a form in which they are capable of bringing about an immune reaction which includes a production of antibodies against the gI.

The present invention is therefore further embodied in novel compositions comprising one or more

oligopeptides/expression products according to this invention brought into an immunogenic form. As well known to those skilled in the art, there are various methods of bringing a substance which is non-immunogenic per se into an immunogenic form. A first possibility is for the oligopeptides/expression products according to this invention to be coupled to a suitable carrier protein. For a chemical coupling, the C-terminus or N-terminus can be used for this purpose. It is well known to those skilled in the art what coupling methods and what carrier proteins are eligible. By way of example, we mention here the possibility of coupling the oligopeptide by means of a suitable coupling agent to KLH (keyhole limpet hemocyanin) or to BSA (bovine serum albumin). Toxoids and liposomes are also suitable carriers. If desired, the oligopeptide is provided at the N-terminus or at the C-terminus with extra amino acid suitable for such a coupling. According to the invention, compositions comprising an immunogenic conjugate of a protein and an oligopeptide according to the invention are primarily preferred. Another possibility is for the oligopeptide to be converted by cross-linking into some larger complex, or for the oligopeptide to be expressed by means of recombinant DNA engineering as a part of a (larger) protein. The invention is accordingly also embodied in compositions comprising an immunogenic complex or an immunogenic recombinant protein comprising an oligopeptide according to the invention.

The oligopeptides/expression products according to the invention may be used in combination with one or more carriers and/or adjuvants suitable for immunization purposes to ensure a strong immune response. Such carriers and adjuvants are known per se. Carriers such as poly-L-lysine, poly-L-glutamic acid, muramyldipeptide and murabutidine can be included in the composition.

Accordingly, the invention is naturally also embodied in novel compositions comprising one or more oligopeptides/expression products according to this invention

in a form suitable for generating an immune response, whereby antibodies are formed, in combination with at least one immuno adjuvant. Suitable immuno adjuvants are known to those skilled in the art. Among the suitable adjuvants are also, for example, aluminium hydroxide and other known adjuvants. Diluents, such as distilled water, phosphate-buffered saline solutions, and buffer solutions (such as a citrate buffer), suitable for the administration of the compositions, are also known per se.

The invention also relates to the use of one or more oligopeptides/expression products according to the invention, or of one or more polyclonal or monoclonal antibodies obtained with the use of the oligopeptides/expression products for diagnostic purposes. More specifically, the invention includes a test of pig sera by means of a set of oligopeptides/expression products according to this invention to determine whether the sera are from a pig infected with the ADV. Such tests can be instrumental to the control or eradication of the ADV in infected pig populations. Techniques suitable for such tests are known per se. We mention here by way of example ELISAs, RIPAs, Western blots and dotblots. According to a more concrete example, porcine blood protected against coagulation is contacted with a monoclonal antibody against pig erythrocytes, to which one of the above-described oligopeptides/expression products according to the invention is coupled. When the blood contains antibodies against the oligopeptide/expression product, an agglutination of the pig erythocytes will take place. In the absence of such antibodies against the oligopetide/expression product, no agglutination will occur.

Porcine serum can also be contacted with a nitrocellulose strip on which various oligopeptides/expression products according to the invention (in a purified or non-purified form) have been arranged. By successively washing, adding a labelled (e.g. peroxidase-coupled) anti-pig antibody, washing again, and adding a substrate for the peroxidase, colouring

can be obtained in the position of the oligopeptide, with which the antibodies present in the sample react.

The invention is illustrated in and by the following description of the tests carried out and the accompanying drawings. As regards the methodology and measuring methods used, reference is made, for the sake of brevity, to the above-cited publications by Geysen et al. The study included competitive ELISAs, immunoscreening of prokaryotic expression products and PEPSCAN analysis to trace reactive peptides of gI of the ADV. The result was that free binding sites for monoclonal antibodies against gI were found to be located in the N-terminal part of gI, between the amino acids 52 and 85. Accordingly, this part of the gI is an antigenic site on which several epitopes are located. As a consequence, there is a great chance that such a site is recognized by polyclonal antisera. This hypothesis was tested by investigating antisera of pigs which in the known gI-ELISA (Van Oirschot et al., 1988) were positive or negative in an indirect ELISA with oligopeptide 52-85 as an antigen. The sera were tested in the peptide ELISA in a 1 : 10 dilution. Eight positive sera were tested, of which five were field sera and three sera of a pig first vaccinated with a gI-negative vaccin and after three weeks challenged with virulent wild-type NIA-3 virus. The sera were collected at days 15, 18 and 21 after the challenge. Of the six negative sera, five came from the field, and one from an spf pig. The gI-seropositive sera all reacted with the oligopeptide, unlike the gI-seronegative sera. The results showed that pigs infected experimentally or in the field form antibodies specifically reacting with the oligopeptide used.

Another result of the study was that two binding sites for monoclonal antibodies against gI were found in the N-terminal part of gI, namely between the amino acids 78 and 238. On the ground of the results of the "conventional" gI-ELISA (articles by Van Oirschot et al.) it was found that the epitopes located in this part are highly immunogenic to the pig. Antibodies against one or both epitopes remain

demonstrable very long after the infection, presumably even during the entire lifetime of the animal. For this reason the invention is not limited to the oligopeptide 52-85, but includes the oligopeptide/expression product 78-238, and more generally all oligopeptides/expression products based on the amino acids 52-238 of gI which are immunologically functional.

Experiments conducted

Monoclonal antibodies

In the study, use was made of a panel of eleven different monoclonal antibodies (MAbs), raised against the pseudorabies virus strains NIA-3 and Phylaxia. The MAbs were purified from ascites fluid by ammonium sulphate precipitation and diluted in phosphate-buffered saline to an ultimate protein concentration of about 7 mg/ml. The MAbs were conjugated with horse radish peroxidase (HRPO) essentially as described by Wilson and Nakane (in the book by Knapp, Holibar and Wick, eds, "Immunofluorescence and related staining techniques", Elsevier, Biochemical Press, Amsterdam, 1978, 215-224).

Competitive ELISA

Dilution series of MAbs-HRPO conjugates in phosphate-buffered saline containing 0.05% Tween 80 were made to determine the highest dilution of conjugate which gives an OD_{450} of 1.5-2.0 in the absence of a second non-labelled antibody. Twice that concentration of conjugate was used in the competitive ELISA. Each of the competing unconjugated MAbs were diluted 1 : 50, 1 : 100, and 1 : 1000 in each of the MAb-HRPO dilutions. The mixtures were transferred to the wells (100 µl/well) of an ELISA plate coated with PRV-antigen. After incubation for 1 h at 37°C, the plates were washed with 0.05% Tween in deionized water, and 100 µl substrate (3,3',5,5'-tetramethylbenzidine or TMB, 1 mg/ml) was added. The reactions were stopped by adding 100 µl 0.5 M H_2SO_4, and the plates were read at 450 nm in a TITERTEK multiscan plate reader. The OD_{450} value in wells without competing antibody was

set at 100%. A MAb dilution which decreased the OD_{450} value of the conjugated MAb by >50% was regarded as competitive.

Topographic analysis of the antigenic domains of gI

The gI specificity of the MAbs was determined by a radio immuno precipitation assay and by SDS-PAGE (results not shown). In the competitive ELISA all MAbs were found to inhibit the binding of the homologous MAb-HRPO by more than 50% (Fig. 1). Using a 1 : 1000 dilution of the competing MAbs, six antigenic domains could be distinguished: antigenic domain A, represented by MAbs 1, 3 and 5; antigenic domain B, represented by MAbs 4, 8 and 11; antigenic domain C, represented by MAbs 6 and 9; antigenic domain D, represented by MAb 7; antigenic domain E, represented by MAb 2, and antigenic domain F, represented by MAb 10. MAb 9 competed for the binding of MAb 6-HRPO in a 1 : 1000 dilution of the competing MAb, whereas MAb 6 competed with conjugated MAb 9 only in a 1 : 50 dilution. Reciprocal competition between MAb 7 and the MAbs 1, 3 and 5 was only effected in a 1 : 50 dilution of the competing MAb. These results indicate that antigenic domains A and D are closely linked. Reciprocal competition was also found between MAbs 4 and 1 in a 1 : 50 dilution. MAbs 3 and 4 competed non-reciprocally only in a 1 : 50 dilution.

Recombinant DNA techniques

Unless mentioned otherwise, the recombinant DNA techniques were carried out essentially as described by Maniatis (in "Molecular Cloning: a Laboratory Manual", Cold Spring Harbor Laboratory, USA, 1982) or by Davis et al. (in "Basic Methods in Molecular Biology", Elsevier, New York, 1986). Restriction enzymes and DNA modifying enzymes were used as prescribed by the manufacturer. Plasmid DNA was prepared using the alkaline lysis method (Maniatis 1982). DNA restriction fragments were isolated from agarose gels by electroelution.

Construction of recombinant plasmids

The pEX plasmids express inserted genes in the form of a cro-B-galactosidase fusion protein. The expression of this gene is under the control of the lambda Pr promotor and is induced by inactivation of the temperature-sensitive cI repressor at 42°C. Escherichia coli bacteria (strain pop 2136, Intitut Pasteur, Paris) were transformed after a thermal shock of 5 min at 34°C and 2 min on ice. Plasmids linearized by a single digestion were dephosphorylated with calf intestine phosphatase. The 2055 bpp AhaIII-NruI fragment (isolated from the BamHI-7 fragment of PRV strain NIA-3, see Quint et al., J.Gen.Virol. 68, 1987, 523-534) with the full gI coding sequence thereon, and fragments derived from the gI coding region were cloned into the correct reading frame into the SmaI site of pEX1, pEX2, or pEX3. Fragments were made blunt if neceesary by a treatment with the Klenow fragment of DNA polymerase I.

Isolation of expression products

The expression of the pEX fusion proteins was induced by incubation of a 1.5 ml exponential culture (OD_{600} about 0.25) of cells at 42°C for 90 min. The expression products were purified as follows: cells were spun down (5 min; 6000xg), re-suspended in 100 µl 50 mM Tris-HCl (pH 8.0), 50 mM EDTA, 15% (w/v) sucrose and treated with lysozyme (1 mg/ml) for 10 min. After the addition of 140 µl 0.2% (w/v) Triton X-100 in 10 mM Tris-HCl (pH 8), the DNA was degraded by adding 24 µl 1 M $MgCl_2$ and 1 µl DNAse (10 mg/ml): Incubation was at 37°C until the suspension was no longer viscous. After the insoluble expression proteins had been spun down, the pellet was resuspended in 250 µl phosphate-buffered saline.

Immunoscreening of expression products

A 5 µl sample of resuspended proteins was dissolved in lysis buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 10% glycerol,

2% SDS, 5% 2-mercaptoethanol, Laemmli, Nature 227, 1970, 680-681). The proteins were fractionated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE, 7.5%), followed by Western blotting using the LKB-multiphor II Nova Blot system in 40 mM glycine, 50 mM Tris, 0.04% SDS (w/v) and 20% methanol (v/v). The nitrocellulose sheets were washed twice in phosphate-buffered saline containing 0.5% (w/v) gelatin, 0.1% (w/v) Triton X-100 (PBS-GT), and incubated with antibodies (MAb 1 : 1000 diluted in PBS-GT) at room temperature for 1 h. The filters were washed twice in PBS-GT for 5 min. and incubated with rabbit anti-mouse IgG-HRPO at room temperature for 1 h. The filters were washed twice in PBS-GT for 5 min. and once in phosphate-buffered saline for 5 min., and incubated in substrate solution (3,3'-diamino-benzidine 0.5 mg/ml and 0.001% H_2O_2) for approximately 5 min.

Localization of antigenic domains by binding of gI-MAbs to gI-specific fusion proteins

An AhaIII-NruI fragment containing the entire gI coding region was cloned in the proper reading frame into pEX2 plasmid to produce the plasmid p1 (see Fig. 2) which contained an insert coding for 685 amino acids. As the insertion was effected at the 3' end of the cro-lac Z gene, expression resulted in a hybrid protein of 115 kDa (cro-B-galactosidase) plus the product of the inserted foreign gene. The predicted size of the expression protein of plasmid p1 is 115+80 kDa (see Mettenleiter et al., J. Virol. 53, 1985, 52-57). Analysis of the hybrid protein with SDS-PAGE showed a protein band of about 190 kDa (the gel electrophoresis and Western blot results of the hybrid B-galactosidase proteins of the various pEX clones are not shown). In a Western blot, ten of the eleven MAbs tested turned out to recognize the fusion protein of plasmid p1 (Table 1).

To map the antigenic domains on the protein, the AhaIII-NruI fragment was treated with NaeI, whereafter the resulting fragments were cloned, depending on the desired

reading frame, in the SmaI site of pEX1, pEX2, or pEX3. Thus the plasmids p2 (with the sequence coding for the amino acids 52 to 305), p3 (with the sequence coding for the amino acids 501 to 654), p4 (with the sequence coding for the amino acids 305 to 411), p5 (with the sequence coding for the amino acids 411 to 501), and p6 (with the sequence coding for the amino acids -15 to 52) were obtained. Only the fusion protein of plasmid p2 turned out to be recognized by the same MAbs that bound to the fusion protein of plasmid p1 (containing the entire gI coding sequence). These results indicated that the antigenic domains A, B, C, D and E were situated between the amino acids 52 and 305. Subsequently, fragments derived from the gI sequence of p2 were cloned in pEX (Fig. 2). In a Western blot, the same 10 MAbs that recognized the fusion proteins of the plasmids p1 and p2 reacted with the fusion proteins of the plasmid ps1 (containing the sequence coding for the amino acids 52 to 238). The MAbs 1, 3 and 5 (antigenic domain A), MAbs 4, 8 and 11 (antigenic domain B) and MAb 7 (antigenic domain D) reacted with the fusion proteins of the plasmid ps4 (containing the sequence coding for the amino acids 52 to 123), but not with the fusion proteins of the plasmids ps2 (containing the sequence coding for the amino acids 78 to 238) and ps3 (with the sequence coding for the amino acids 123 to 238). MAb 2 (antigenic domain E) and MAbs 6 and 9 (antigenic domain C) only reacted with the fusion protein of plasmid ps2. The fusion protein of plasmid ps3 was not recognized by any of the tested gI MAbs (see Table 1). These findings indicate that the antigenic domains A, B and D are located between the amino acids 52 and 78, and that the antigenic domains C and E are located between the amino acids 78 and 238.

PEPSCAN

Overlapping nonapeptides covering amino acids 52-238 of the gI protein were synthesized and tested as described by

Geysen et al. All MAbs were tested in the ELISA in a dilution of 1 : 150.

Localization of the epitopes with the PEPSCAN method

The 168 overlapping nonapeptides spanning the amino acids 52 to 238 of the ps1 plasmid were tested by the PEPSCAN method, using the gI MAbs. MAbs 1, 3 and 5 (antigenic domain A) reacted in the same way with peptides within the amino acid sequences 63-73 and 75-84. MAbs 4, 8 and 11 (antigenic domain B) reacted in the same way with peptides within the amino acid sequence 52-67. MAb 7 (antigenic domain D) recognized peptides within the amino acid sequence 68-84 (see Fig.3). MAbs 2, 6, 9 and 10, representing the antigenic domains C, E and F, did not react with any of the peptides investigated.

The results indicate that antigenic domain D is a continuous domain, whereas the antigenic domain A and B are semi-continuous. The fact that, according to the immunoscreening, the antigenic domains C and E (belonging to the MAbs 2, 6 and 9) should be located on plasmid ps2, but the MAbs in question do not react with the fusion proteins of the plasmids ps3 and ps4, or with any nonapeptide in the PEPSCAN analysis appears to indicate that a local conformation is required for the recognition between these MAbs and their respective epitopes, which local conformation is present in the fusion protein of plasmid ps2, but not in the fusion proteins of plasmids ps3 and ps4, or in the synthetic nonapeptides.

Description of the drawings

Fig. 1 shows the results of the competitive ELISA.

Fig. 2 shows the cloning strategy of the entire sequence coding for gI and fragments thereof in pEX expression plasmids. The fat line indicates the open reading frame of gI. The numbers indicate amino acid positions.

Fig. 3 shows the results of the PEPSCAN analysis of the 33 nonapeptides covering the amino acids 52 to 84. The amino

acid sequence is indicated in the one-letter code, with reference to the amino acid position. The horizontal lines below the amino acids sequence mark the peptides binding to the MAbs indicated. The fat lines indicate the peptides binding strongest to the MAbs. The vertical lines indicate the OD value resulting from the reaction of the MAbs with the nonapeptides.

Table 1: reactivity of MAbs with pEX expression proteins containing gI fragments and in the PEPSCAN analysis

pEX clones							PEPSCAN
MAb	p1	p2	ps1	ps2	ps3	ps4	
1	+	+	+	-	-	+	+
2	+	+	+	+	-	-	-
3	+	+	+	-	-	+	+
4	+	+	+	-	-	+	+
5	+	+	+	-	-	+	+
6	+	+	+	+	-	-	-
7	+	+	+	-	-	+	+
8	+	+	+	-	-	+	+
9	+	+	+	+	-	-	-
10	-	-	-	-	-	-	-
11	+	+	+	-	-	+	+

CLAIMS

1. Oligopeptide having the following amino acid sequence (according to the three-letter code)
Ala Gly Asp Asp Asp Leu Asp Gly Asp Leu Asn Gly Asp Asp Arg
Arg Ala Gly Phe Gly Ser Ala Leu Ala Ser Leu Arg Glu Ala Pro
Pro Ala His Leu Val Asn Val Ser Glu Gly Ala Asn Phe Thr Leu
Asp Ala Arg Gly Asp Gly Ala Val Val Ala Gly Ile Trp Thr Phe
Leu Pro Val Arg Gly Cys Asp Ala Val Ala Val Thr Met Val Cys
Phe Glu Thr Ala Cys His Pro Asp Leu Val Leu Gly Arg Ala Cys
Val Pro Glu Ala Pro Glu Arg Gly Ile Gly Asp Tyr Leu Pro Pro
Glu Val Pro Arg Leu Gln Arg Glu Pro Pro Ile Val Thr Pro Glu
Arg Trp Ser Pro His Leu Thr Val Arg Arg Ala Thr Pro Asn Asp
Thr Gly Leu Tyr Thr Leu His Asp Ala Ser Gly Pro Arg Ala Val
Phe Phe Val Ala Val Gly Asp Arg Pro Pro Ala Pro Leu Ala Pro
Val Gly Pro Ala Arg His Glu Pro Arg Phe His Ala Leu Gly Phe
His Ser Gln Leu Phe Ser Pro
which sequence corresponds to that of the amino acids 52-238 of the glycoprotein I (gI) of the Aujeszky's disease virus (ADV), or an immunologically functional fragment or derivative thereof.

2. An oligopeptide according to claim 1, having the following amino acid sequence (according to the three-letter code)
Ala Gly Asp Asp Asp Leu Asp Gly Asp Leu Asn Gly Asp Asp Arg
Arg Ala Gly Phe Gly Ser Ala Leu Ala Ser Leu Arg Glu Ala Pro
Pro Ala His Leu
which sequence corresponds to that of the amino acids 52-85 of gI of ADV, or an immunologically functional fragment or derivative thereof.

3. An oligopeptide according to claim 2, and having the following amino acid sequence (according to the three-letter code)
Ala Gly Asp Asp Asp Leu Asp Gly Asp Leu Asn Gly Asp Asp Arg
Arg

which sequence corresponds to that of the amino acids 52-67 of gI of ADV, or an immunologically functional fragment or derivative thereof.

4. An oligopeptide according to claim 2, and having the following amino acid sequence (according to the three-letter code)

Asn Gly Asp Asp Arg Arg Ala Gly Phe Gly Ser Ala Leu Ala Ser Leu Arg Glu Ala Pro Pro Ala His Leu

which sequence corresponds to that of the amino acids 62-85 of gI of ADV, or an immunologically functional fragment or derivative thereof.

5 An oligopeptide according to claim 4, having the following amino acid sequence (according to the three-letter code)

Ala Gly Phe Gly Ser Ala Leu Ala Ser Leu Arg Glu Ala Pro Pro Ala His Leu

which sequence corresponds to that of the amino acids 68-85 of gI of ADV, or an immunologically functional fragment or derivative thereof.

6. An oligopeptide having the following amino acid sequence (according to the three-letter code)

Arg Glu Ala Pro Pro Ala His Leu Val Asn Val Ser Glu Gly Ala Asn Phe Thr Leu Asp Ala Arg Gly Asp Gly Ala Val Val Ala Gly Ile Trp Thr Phe Leu Pro Val Arg Gly Cys Asp Ala Val Ala Val Thr Met Val Cys Phe Glu Thr Ala Cys His Pro Asp Leu Val Leu Gly Arg Ala Cys Val Pro Glu Ala Pro Glu Arg Gly Ile Gly Asp Tyr Leu Pro Pro Glu Val Pro Arg Leu Gln Arg Glu Pro Pro Ile Val Thr Pro Glu Arg Trp Ser Pro His Leu Thr Val Arg Arg Ala Thr Pro Asn Asp Thr Gly Leu Tyr Thr Leu His Asp Ala Ser Gly Pro Arg Ala Val Phe Phe Val Ala Val Gly Asp Arg Pro Pro Ala Pro Leu Ala Pro Val Gly Pro Ala Arg His Glu Pro Arg Phe His Ala Leu Gly Phe His Ser Gln Leu Phe Ser Pro

which sequence corresponds to that of the amino acids 78-238 of gI of ADV, or an immunologically functional fragment or derivative thereof.

7. An oligopeptide according to any one of claims 1-6 in the form of an expression product obtained by expression of genetic information coding for the amino acid sequence of the oligopeptide.

8. A composition comprising at least one oligopeptide or immunologically functional fragment or derivative thereof as claimed in any one of claims 1-7, brought into an immunogenic form.

9. A composition as claimed in claim 8, comprising an immunogenic conjugate of a protein and an oligopeptide or immunologically functional fragment or derivative thereof as claimed in any one of claims 1-7.

10. A composition as claimed in claim 8, comprising an immunogenic complex including an oligopeptide or immunologically functional fragment or derivative thereof as claimed in any one of claims 1-7.

11. A composition as claimed in claim 8, comprising an immunogenic recombinant protein including an oligopeptide or immunologically functional fragment or derivative thereof as claimed in any one of claims 1-7.

12. A recombinant polynucleotide comprising a nucleotide sequence coding for an oligopeptide or immunologically functional fragment or derivative thereof according to any one of claims 1-7.

13. Recombinant DNA comprising a DNA sequence coding for an oligopeptide or immunologically functional fragment or derivative thereof according to any one of claims 1-7.

14. Recombinant DNA consisting of a vector portion and an insertion portion containing a DNA sequence coding for an oligopeptide or immunologically functional fragment or derivative thereof according to any one of claims 1-7.

15 A host cell genetically engineered with the help of a recombinant DNA according to claim 14 to be given the capacity of expressing an oligopeptide or immunologically functional fragment or derivative thereof according to any one of claims 1-7.

16. Microorganisms genetically engineered with the help of a recombinant DNA according to claim 14 to be given the capacity of expressing an oligopeptide or immunologically functional fragment or derivative thereof according to any one of claims 1-7.

17. Bacteria genetically engineered with the help of a recombinant DNA according to claim 14 to be given the capacity of expressing an oligopeptide or immunologically functional fragment or derivative thereof according to any one of claims 1-7.

18. Eukaryotic cells genetically engineered with the help of a recombinant DNA according to claim 14 to be given the capacity of expressing an oligopeptide or immunologically functional fragment or derivative thereof according to any one of claims 1-7.

19. The use of an oligopeptide or immunologically functional fragment or derivative thereof as claimed in any one of claims 1-7 for diagnostic purposes.

20. The use of an oligopeptide or immunologically functional fragment or derivative thereof as claimed in any one of claims 1-7 for serologically distinguishing animals infected by ADV from animals not infected by ADV.

21. The use of an oligopeptide or immunologically functional fragment or derivative thereof as claimed in any one of claims 1-7 for serologically distinguishing pigs infected by ADV from pigs not infected by ADV.

22. The use of an oligopeptide or immunologically functional fragment or derivative thereof as claimed in any one of claims 1-7 for producing polyclonal or monoclonal antibodies having a specific affinity to gI of ADV.

23. A kit for diagnostic examination for ADV infection, comprising an oligopeptide or immunologically functional fragment or derivative thereof as claimed in any one of claims 1-7.

24. The use of polyclonal or monoclonal antibodies having a specific affinity to gI of ADV, and obtained using an

oligopeptide or immunologically functional fragment or derivative thereof as claimed in any one of claims 1-7, for diagnostic purposes.

25. A kit for a diagnostic test for ADV infection comprising polyclonal or monoclonal antibodies having a specific affinity to gI of ADV, and obtained using an oligopeptide or immunologically functional fragment or derivative thereof as claimed in any one of claims 1-7.

INTERNATIONAL SEARCH REPORT

International Application No PCT/NL 90/00143

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: C 07 K 15/14, C 12 N 15/38, G 01 N 33/569, A 61 K 39/245

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[5]	C 12 N, A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
A	EP, A, 0223382 (THE UPJOHN COMPANY) 27 May 1987 see pages 34-36 (cited in the application) --	1-25
A	Virology, volume 171,no. 2, August 1989, Academic Press, Inc., T.A. Kost et al.: "Biological evaluation of glycoproteins mapping to two distinct mRNAs within the BamHI fragment 7 of pseudorabies virus: expression of the coding regions by vaccinia virus", pages 365-376 see the whole article --------	1-25

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
8th January 1991	29. 01. 91
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	Natalie Weinberg

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. NL 9000143 SA 40684

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 18/01/91
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0223382	27-05-87	AU-B- 601378	13-09-90
		AU-A- 6375186	24-04-87
		EP-A- 0238618	30-09-87
		JP-T- 63501611	23-06-88
		WO-A- 8702058	09-04-87